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Dysregulation of miR-146a in human milk of mothers having children with autism

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Abstract

Autism spectrum disorder (ASD) is a set of neurobehavioral manifestations that impose poor social interaction and stereotyped repetitive patterns. Several mircoRNA (miRNA) dysregulations underpin ASD pathophysiology via impairing the neurogenic niches. For instance, miR-146a and miR-106 differential expressions are linked to deregulation of ASD-related genes and the severity of clinical symptoms, respectively. Breastfeeding provides newborns with many bioactive compounds that support their neurodevelopment including miRNA. Our pilot study evaluated the expression pattern of miR-106a and miR-146a in human milk (HM) of nursing mothers ($n = 36$) having autistic children compared to age-matched counterparts ($n = 36$) with neurotypical children as controls. Under sterile conditions, breast milk samples were collected using manual sucking pumps and centrifuged to separate the fat layer. Total RNA was extracted from the lipid fraction, and the expression profiles of both miR-106a and miR-146a were evaluated using quantitative real-time polymerase chain reaction. Among the test group, we reported some factors that were previously linked to HM miRNA perturbations: gestational diabetes, hypertension, and cesarean delivery. HM miR-106a showed comparable expression levels in both mother groups ($p = 0.8681$), whereas HM miR-146a was significantly downregulated in mothers with autistic children compared to controls ($p = 0.0399$). Alternatively, HM miR-106 levels were positively associated with two ASD clinical parameters: Childhood Autism Rating Scale (CARS) and communication and language domain of Autism Diagnostic Interview-Revised (ADI-R) ($r = 0.6452$, $p = 0.0003$ and $r = 0.3958$, $p = 0.0410$, respectively). The receiver operating characteristic (ROC) curves of both maternal HM miR-106a and miR-146a showed poor fitness as predictive biomarkers for ASD. Our findings suggest that the miR-146a differential expression in ASD children may originate at infancy during the lactation period.

Abbreviations: 3′ UTRs, 3′ untranslated regions; ADI-R, Autism Diagnostic Interview-Revised; APA, American Psychiatric Association; ASD, autism spectrum disorder; AUC, area under the curve; CARS, Childhood Autism Rating Scale; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; HM, human milk; miR, microRNA; miRNA, microRNA; NAD, no abnormality detected; qRT-PCR, quantitative real-time polymerase chain reaction; ROC, receiver operating characteristic; SEM, standard error of the mean; SHANK, SH3 and multiple ankyrin repeat domains.

Thus, maternal pre- and postnatal health care is critical to maintain optimal miRNome in breast milk.

KEYWORDS
autism spectrum disorder, human milk, microRNA, miR-106a, miR-146a

1 | INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by poor communication and social interaction as well as inflexible and repetitive behaviors (American Psychiatric Association [APA], 2013). It affects about 1% of children worldwide (Zeidan et al., 2022). The etiology of ASD is rather complex with multiple genetic and environmental determinants. Neurodegenerative events, such as protein disaggregation, have also been implicated in ASD development (Jęśko et al., 2020). Epigenetics factors—including microRNA (miRNA)—represent additional confounders of ASD (Eshraghi et al., 2018). Generally, miRNAs regulate the expression of specific genes mediating the developmental processes in central nervous system from early neurogenesis to synapse formation (Cho et al., 2019). In ASD, miRNA-associated mutations occur within (a) miRNA-encoding genes, (b) pre-miRNAs and adjacent regions, and/or (c) 3′ untranslated regions (3′ UTRs) of miRNA target genes. The mutated 3′ UTR of mRNA may perturb miRNA-mediated regulation and ultimate abnormal expression of ASD-related genes (Li et al., 2022). Previous studies investigated the expression profile of over 218 miRNAs in individuals with ASD and reported several alterations. The dysregulation of miR-106 family is one of the most common molecular hallmarks in ASD. Moreover, the altered circulating levels of miR-106a were associated with ASD manifestations (Garrido-Torres et al., 2023). At the cellular level, the overexpression of miR-146a deregulates up to 40% of the ASD brain transcriptome (Nguyen et al., 2018). Therefore, numerous studies suggested miRNAs as predictive biomarkers for neurodevelopmental disorders including ASD (Juvale & Che Has, 2021).

Alternatively, many lines of evidence linked maternal risk factors to ASD onset in offspring. For example, pre-existing and gestational diabetes increase the risk for having autistic children by 62% and 42% respectively. Other maternal health conditions showed potential contribution to ASD risk such as polycystic ovary syndrome, hypertension, and obesity (Lu et al., 2022). Microbial infections and immune activation represent another possible causality, where maternal brain-reactive antibodies can account for nearly 20% of ASD cases (Bagnall-Moreau et al., 2023). Similar outcomes were observed in cohort studies involving pregnant or lactating women who consume antiepileptic drugs such as valproic acid, oxcarbazepine, and lamotrigine (Veroniki et al., 2017). Besides, in utero exposure to pollutants, particularly organophosphates, was associated with 60% increase in ASD diagnosis among offspring (Shelton et al., 2014). Maternal dietary deficiencies during pregnancy or breastfeeding can also impair the neurodevelopment of their infants. Adequate intake of folic acid and vitamin D during the periconceptional and prenatal periods, respectively, can reduce the likelihood of giving birth to children with ASD (Zhong et al., 2020). Lastly, partial or exclusive breastfeeding showed protective effect against ASD. Toddlers who have not been breastfed during their first six months were 2.34 times more likely to be diagnosed with ASD (Huang et al., 2021).

Besides its nutritional components, human milk (HM) contains many bioactive compounds such as immunoglobulins, prebiotics, and growth factors. Several studies detected endogenous non-coding RNAs in HM including circular RNA, transfer RNA, and ribosomal RNA among others (Tingö et al., 2021). It is also one of the richest reservoirs of miRNAs among human biofluids (Kim & Yi, 2020). HM miRNA mainly originates from the mammary glands with a small portion flowing from the maternal bloodstream. Hence, mothers show unique miRNA profile in breast milk compared with blood cells and plasma (Alsaweed et al., 2016). HM miRNAs are transported in nano-sized exosomes (exomiRs) surrounded by a double-layered lipid membrane to facilitate its survival amid the gastrointestinal environment and systemic circulation in the newborn (Chiurazzi et al., 2021). The miRNA content varies across HM fractions with cellular and lipid partitions having greater abundance (Alsaweed et al., 2015). Moreover, HM miRNome involve dynamic miRNAs that fluctuate throughout the lactation period (Raymond et al., 2022). Maternal health conditions (e.g., diabetes and obesity) can also contribute to differential expression of specific miRNA species (Mirza et al., 2019; Shah et al., 2021). Preterm/full term and mode of delivery influence the miRNA composition of HM (Carney et al., 2017). For

instance, vaginal delivery induces endogenous oxytocin that, in turn, modulates the expression and secretion of miR-148a and miR-320 in colostrum milk (Gutman-Ido et al., 2022).

Growing evidence uncovered the essential role of HM-derived miRNA in the neurodevelopment of infants (Chiurazzi et al., 2021). Half of mammalian miRNAs involved in synaptogenesis overlaps with the top 288 exomiRs present in HM (Lönnerdal, 2019). Therefore, we assumed that HM miRNA profile can act as an additional maternal risk factor contributing to ASD onset among offspring. Using quantitative real-time polymerase chain reaction (qRT-PCR), our study investigated milk samples from mothers with autistic children to quantify the levels of two ASD-related miRNAs: miR-106a and miR-146a.

2 | MATERIALS AND METHODS

2.1 | Subjects

This study was approved by the Medical Research Ethics Committee, National Research Centre in Egypt (Ethical Approval Number: 01441223) in accordance with Helsinki Declaration. Written informed consent was obtained from each participant. Thirty-six women who have children with ASD were included in the current pilot study as well as 36 healthy women with neurotypical children as a control group. Subjects were selected from those attending the outpatient clinic of Children with Autistic Disorder, Medical Research Center of Excellence, National Research Centre, Egypt. All participating mothers were lactating a healthy infant at the time of recruitment. Full clinical history and pedigree analysis were recorded. The children with ASD were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DMS-5) (APA, 2013), Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994), and Childhood Autism Rating Scale (CARS) (Schopler et al., 2010).

2.2 | Sampling

Fifteen milliliters of breast milk were collected from each mother in RNase-free falcon tube using a sterile manual sucking pump. Within 30 min, the fresh milk samples were centrifuged at 720 *g* for 20 min at 4°C (Haraeus, Labofuge 400R; Germany) to fractionate the milk (Alsaweed et al., 2015). The fat layer was then transferred to a new nuclease-free 1.5-ml Eppendorf tube for RNA extraction.

2.3 | qRT-PCR

Total RNA was extracted from each milk sample using miRNeasy Mini Kit (Qiagen, catalog number: 217004), according to the manufacturer's instructions. RNA concentration and purity were checked using nanodrop spectrophotometer (2000 C, Thermo Scientific, USA). RNA samples were stored at −80°C until further analysis. The reverse transcription reaction was performed using miScript II RT Kit (Qiagen, catalog number: 218161). qRT-PCR was performed using Rotor Gene Q (Qiagen, USA) and primer assays specific for Hs miR-106a*_1 (Qiagen, catalog no. MS00008393) and Hs miR-146a_1 (Qiagen, catalog no. MS00003535). The reaction comprised 4 µl of cDNA (100 ng/µl), 2 µl of 10x miScript primer assay, 2 µl of 10x miScript Universal Primer, and 10 µl of 5x Quantitect SYBR Green PCR Master Mix; the total reaction volume was completed up to 20 µl using nuclease-free water. The thermal cycling profile was set as follows: initial denaturation at 95°C for 15 min followed by 40 cycles at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Hs miR-RNU6-2_11 (Qiagen, catalog number: MS00033740) was used as an internal control to normalize the cycle threshold (Ct) values (Munch et al., 2013). Fold change was calculated using the 2^(−ΔΔCt) method (Schmittgen & Livak, 2008).

2.4 | Statistical analysis

Data analysis was performed using GraphPad Prism version 6. Mann–Whitney *U* test and Spearman's rank correlation were used to compare the quantitative variables and assess their associations, respectively. Receiver operating characteristic (ROC) curve was plotted to evaluate the ASD risk prediction efficacy of HM miR-106a and miR-146a. Quantitative data are represented as mean ± standard error of the mean (SEM), whereas qualitative and categorical data are represented as proportion and percentage. The level of significance was set at 5%.

3 | RESULTS

3.1 | The sociodemographic and clinical data

A total of 72 breastfeeding women were recruited including 36 mothers with autistic children and 36 age-matched counterparts with neurotypical children as controls. Table 1 shows the descriptive data of the mother and autistic child dyad in the test group. Five pregnancy

TABLE 1 Sociodemographic and clinical data of mothers and their autistic children. Qualitative and quantitative data are represented as proportion (percentage) and mean ± SEM, respectively.

Mothers ($n = 36$)	
Age (years)	31.59 ± 0.81
Consanguinity (positive)	13/36 (36.11%)
Pregnancy history	
NAD	19/36 (52.78%)
Hypertension	5/36 (13.89%)
Oligohydramnios	4/36 (11.11%)
Diabetes	3/36 (8.33%)
Bleeding	3/36 (8.33%)
Hypothyroidism	2/36 (5.56%)
Delivery (cesarean section)	32/36 (88.89%)
Autistic children ($n = 36$)	
Age (years)	5.79 ± 0.52
Gender (male)	27/36 (75%)
Birth order	
First	24 (66.67%)
Second	12 (33.33%)
CARS	34.53 ± 0.53
Severity	
Mild-to-moderate	25/36 (69.44%)
Severe	11/36 (30.56%)
ADI-R	
Reciprocal social interaction	13.89 ± 0.54
Communication and language skills	8.58 ± 0.35
Repetitive, stereotyped, and restricted behaviors and interests	7 ± 0.36

Abbreviations: ADI-R, Autism Diagnostic Interview-Revised; CARS, Childhood Autism Rating Scale; NAD, no abnormality detected; SEM, standard error of the mean.

complications were reported among those mothers: hypertension, oligohydramnios, hypertension, diabetes, bleeding, and hypothyroidism. Of note, over 88% of the mothers who gave birth to a child, later diagnosed with ASD, had a cesarean section. On the other hand, 66.67% of the autistic children were the first in birth order. Based on CARS, they were sub-classified into 25 mild-to-moderate and 11 severe cases.

3.2 | The fold change of HM miR-106a and miR-146a and ROC curve

Unlike miR-106a, the expression level of miR-146a in HM of mothers with ASD children was significantly downregulated compared with controls. ROC curve of miR-146a also showed better predictive accuracy compared with miR-106a as shown in Figure 1.

3.3 | Correlation between HM miR-106a and miR-146a levels and ASD clinical parameters

Despite the significant downregulation of maternal HM miR-146a, it showed negligible correlation with the autistic symptoms in the affected children (Table 2). Conversely, the expression levels of HM miR-106a were positively correlated with two clinical parameters: CARS and the communication and language skills ($p = 0.0003$ and 0.0410, respectively).

4 | DISCUSSION

The etiology of ASD is elusive with interconnected genetic and environmental contributions. miRNAs lie at the interface between both factors to modulate ASD-related gene expressions in response to environmental stimulants (Beversdorf et al., 2021). Meta-analysis reported common miRNA dysregulations in the peripheral and cellular milieu of individuals with ASD (Garrido-Torres et al., 2023). The differential expression of ASD-related *SH3 and multiple ankyrin repeat domains* (*SHANK*) gene family is linked to circulating miR-7 levels among families with neuropsychiatric history (Abdelrahman et al., 2021). Some heritable miRNA dysregulations (e.g., miR-19a-3p, miR-126-3p, and miR-150-5p) have also been identified in autistic children and their parents (Ozkul et al., 2020). Meanwhile, several pre- and postnatal maternal risk factors may additively contribute to ASD onset (Lu et al., 2022). Given its rich availability, the role of maternal HM-derived miRNA in neurodevelopment of neonates is a nascent field of research (Chiurazzi et al., 2021). Differentially expressed HM miRNAs are implicated in transcriptional/translational regulation of gene products responsible for dopaminergic and glutamatergic pathways, neurotransmitter secretion, neuronal morphogenesis, and synaptic vesicle transport (Freiría-Martínez et al., 2023). Breastfeeding a baby for 6 months was linked to a 54% reduction in risk of ASD (Ghozy et al., 2020). Therefore, this study compared the abundance of two ASD-related

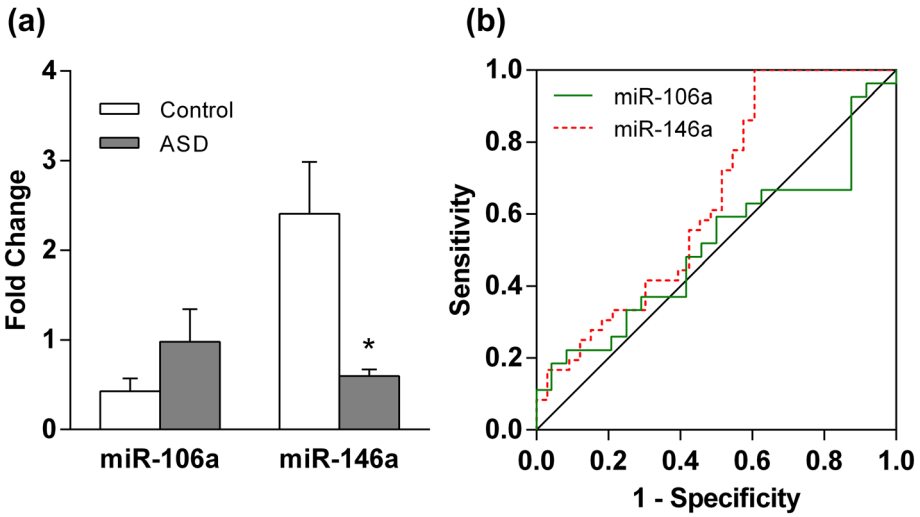

FIGURE 1 (a) The relative abundance of both miRNAs in milk samples from mothers having autistic children ($n = 36$) compared with controls ($n = 36$). miR-106a was upregulated by 2.29 folds ($p = 0.8681$), whereas miR-146a was downregulated by 4.02 folds ($p = 0.0399$). The data are represented as mean ± SEM. (b) Receiver operating characteristic (ROC) curve of maternal human milk (HM) miR-106a and miR-146a to assess their predictive accuracy for autism spectrum disorder (ASD). miR-146a showed better discrimination potential (area under the curve [AUC] = 0.6439, $p = 0.04001$ with 72.22% sensitivity and 48.48% specificity at cutoff 0.9867) compared with miR-106a (AUC = 0.5139, p = 0.8651 with 62.96% sensitivity and 41.67% specificity at cutoff 0.01806).

TABLE 2 Spearman's correlation between fold change of each miRNA and the demographic and clinical parameters.

Factor	miR-106a fold change	miR-146a fold change
Mother's age	$r = 0.007637$; $p = 0.9698$	$r = 0.1435$; $p = 0.4039$
CARS	$r = 0.6452$; $p = 0.0003$	$r = 0.2500$; $p = 0.1414$
ADI-R domains		
Reciprocal social interaction	$r = 0.2506$; $p = 0.2074$	$r = -0.01977$; $p = 0.9089$
Communication and language skills	$r = 0.3958$; $p = 0.0410$	$r = 0.05125$; $p = 0.7666$
Repetitive, stereotyped, and restricted behaviors and interests	$r = -0.06905$; $p = 0.7322$	$r = -0.2031$; $p = 0.2348$

Abbreviations: ADI-R, Autism Diagnostic Interview-Revised; CARS, Childhood Autism Rating Scale; miR, microRNA; *p*, probability value; *r*, correlation coefficient.

miRNAs, namely, miR-106a and miR146a, in the lipid fraction of breast milk samples obtained from mothers having children with and without ASD. It also elucidated their associations with various autistic manifestations.

Alsaweed et al. classified miR-106a among the top ten most highly expressed miRNA species in breast milk fat and cells (Alsaweed et al., 2016). Our results showed comparable levels of HM miR-106a in mothers with or without autistic children. However, we reported a significant positive correlation between its levels and two ASD clinical parameters: CARS and the communication and language domain of ADI-R. This agrees with an earlier study that found that increased circulating levels of miR-106a in children were positively correlated with the severity of autistic symptoms assessed by CARS (Zamil et al., 2020). Another study also reported a direct association between salivary miR-106a-5p levels and the restricted and repetitive behavior in ASD children (Hicks et al., 2020). Our finding, however, disagrees with a study that found no association between breastfeeding in general and the clinical severity of ASD (Peries et al., 2023). Alternatively, milk samples from mothers with ASD children exhibited fourfold less miR-146a content compared with those of control mothers in our study. Despite the remarkable overexpression of miR-146a in ASD brain, prior studies revealed discordant observations with respect to its expression pattern in peripheral blood (Wu et al., 2020). Our finding can be attributed to miR-146a stage-specific downregulation in the human breast milk throughout lactation (Raymond et al., 2022). miR-146a is a strong candidate for ASD pathophysiology (Fregeac et al., 2016). Given its abnormal expression profile in brain cells and body fluids of autistic subjects, it can be a potential diagnostic biomarker (Wu et al., 2020). The ROC curve plotted in our study showed that maternal HM miR-146a provide slightly better discriminative accuracy (area under the curve [AUC] = 0.6439, $p = 0.04001$ with 72.22% sensitivity and 48.48% specificity at cutoff 0.9867) compared with miR-106a (AUC = 0.5139,

$p = 0.8651$ with 62.96% sensitivity and 41.67% specificity at cutoff 0.01806). However, neither one can be suggested as predictive biomarker for ASD given their poor AUC (Safari et al., 2016).

A myriad of maternal factors can alter the expression profile of miRNA in breast milk. These include age, diet, and some diseases (Kondracka et al., 2023). Nonetheless, the maternal age at recruitment was not associated with both HM miR-106a and miR-146a levels in our participants. We reported several pregnancy complications among mothers in the test group including diabetes and hypertension that have been recently linked to HM miRNA dysregulation. Gestational diabetes mellitus altered the levels of HM miR-148a, miR-30b, and miR-let-7 family in affected mothers (Shah et al., 2022). Similarly, women with hypertension exhibited aberrant expression pattern of miR126, miR155, miR21, and miR29a in the cellular fraction of their breast milk (Kondracka et al., 2024). Thus, these health conditions may also contribute to the reported HM miR-146a downregulation. Interestingly, the delivery method can also perturb the miRNA profile in HM (Yerlikaya et al., 2021). About 89% of our study cohort who gave birth to autistic children underwent cesarean section. Yerlikaya et al. implicated cesarean birth to the upregulation of 17 miRNA species in colostrum (Yerlikaya et al., 2021). It also differently modulates the expression of specific miRNAs (e.g., miR-148a and miR-125b) in colostrum compared with mature milk samples (Chiba et al., 2022). Cesarean delivery also diminishes the frequency of early breastfeeding thus potentially perturbing the newborn epigenetic neuronal programming (Słyk-Gulewska et al., 2023). Moreover, differential miRNA expression is evident in breast milk of primiparous compared with multiparous women (Chiba et al., 2022). This is confirmed by our study cohort where over half of the mothers with ASD children were primiparous. Therefore, our data suggest a link of cesarean delivery and parity with miR-146a downregulation in HM lipid fraction that possibly impairs the neurodevelopment of breastfed infants. The identification of potential ASD risk factors allows for prevention and/or early intervention.

5 | CONCLUSION

This study highlights the prospective role of maternal HM miR-146a differential expression in ASD development among offspring. Maternal nutrition, well-being, and healthy pregnancy should be maintained to ensure optimal miRNome in breast milk. We recommend routine prenatal and postnatal maternal health care as a preventive tool. In addition, the identification of potential HM miRNA predictive biomarkers for ASD can allow for early intervention and may be prevention. However, research does suggest a correlation between reduced risk of ASD when breastfeeding. The results should be interpreted cautiously due to the small sample size. The insufficient number of mother participants in this study is attributed to the stringent eligibility criteria. Further profiling is warranted to investigate the expression patterns of HM-derived miRNA panels and their associations with ASD diagnosis and symptoms to emphasize their pivotal role.

ETHICS STATEMENT

This study was approved by the Medical Research Ethics Committee, National Research Centre in Egypt (Ethical Approval Number: 01441223) according to Helsinki Declaration. Written informed consent was obtained from each participant.

AUTHOR CONTRIBUTIONS

Nagwa A. Meguid: conceptualization, study design, clinical investigations, interpretation of data, and writing the manuscript. Maha Hemimi: conceptualization, study design, laboratory experiments, data analysis, interpretation of data, and writing the manuscript. Mahmoud Rashad: conceptualization, study design, and interpretation of data. Amal Elsaeid and Gina Elpatrik: conceptualization, clinical investigations, and curation of data. Hala M. Zeidan: conceptualization, study design, laboratory experiments, data analysis, interpretation of data, and writing the manuscript. All authors reviewed and approved the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

None to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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